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Chromosomal Proteins of the Sperm of a Cephalochordate (*Branchiostoma floridae*) and an Agnathan (*Petromyzon marinus*): Compositional Variability of the Nuclear Sperm Proteins of Deuterostomes

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Abstract. We have isolated and characterized for the first time the chromosomal proteins from the nucleus of the sperm of a lancelet (amphioxus) *Branchiostoma floridae* (Hubbs, 1922) (Phylum Chordata: Subphylum Cephalochordata) and of a lamprey *Petromyzon marinus* (Linnaeus, 1758) (Phylum Chordata: Subphylum Vertebrata: Class Agnatha). In the first case, the major protein component of the sperm-chromatin of a lancelet is a highly specialized protamine-like (PL) protein that has structural and compositional features similar to those of PL-III from bivalve mollusks. In contrast, the chromatin of the sperm of the lamprey has a structural arrangement and protein composition (histones) very similar to that found in the somatic cells of all eukaryotic organisms.

Among the deuterostomes, chromosomal protein variability is considerably greater in representatives of the Phylum Chordata than in echinoderms. The possible evolutionary significance of these findings is discussed.

Introduction

The first nuclear sperm-specific proteins were isolated from vertebrates. They were obtained from the sperm of a salmonid fish *Salmo salar* (Rhine Salmon) and were given the name of protamines (Miescher, 1874; Kossel, 1928). It was clear from the very beginning that these proteins were different "chemically" from the proteins found in the nucleus of somatic cells (Kossel, 1928). Protamines are small (30–40 amino acid) arginine rich (≥50% arginine) proteins that displace the somatic-like spermatogenic histones during spermiogenesis. Besides the early salmonid protamines, other homologous proteins have also been identified in other groups of vertebrates including amphibians (Bols and Kasinsky, 1972, 1973; Kasinsky *et al.*, 1978, 1985; Mann *et al.*, 1982; Takamune *et al.*, 1991), reptiles (Kasinsky *et al.*, 1978; Mann, 1981; Kasinsky *et al.*, 1987; Chiva *et al.*, 1989), birds (Dixon *et al.*, 1985; Chiva *et al.*, 1987, 1988) and mammals (this group has been recently reviewed by Oliva and Dixon, 1991). See also Kasinsky (1989) for an overall review.

Despite their wide distribution and the historical discovery of protamines in vertebrates, the protein composition of the sperm chromatin of this taxonomic group is extremely heterogeneous (Bloch, 1969, 1976; Kasinsky, 1989). Such protein heterogeneity is also shared by other taxonomic groups (Bloch, 1969, 1976). The protein composition of the sperm may thus vary from somatic-type histones (H) to compositionally intermediate protamine-

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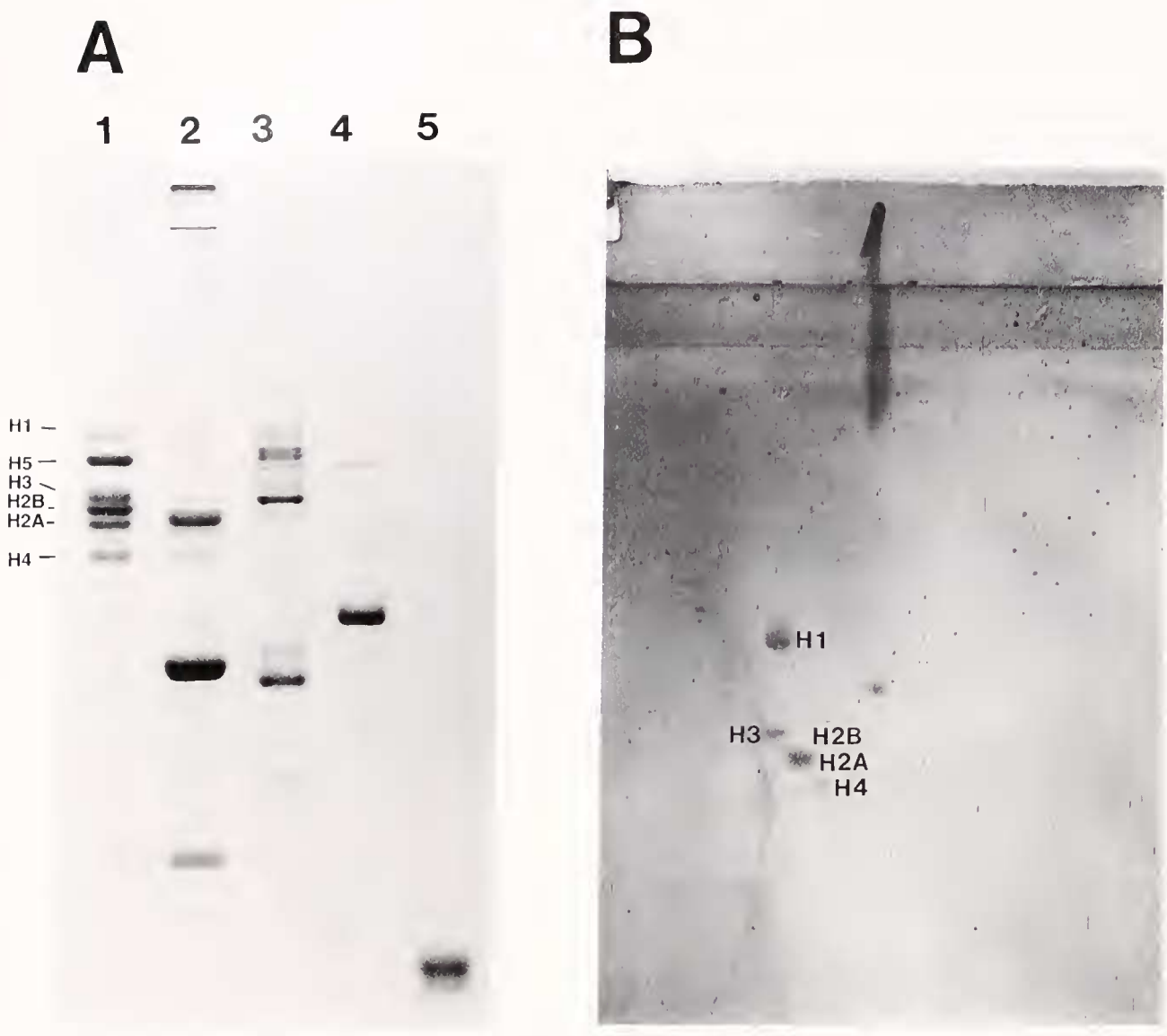
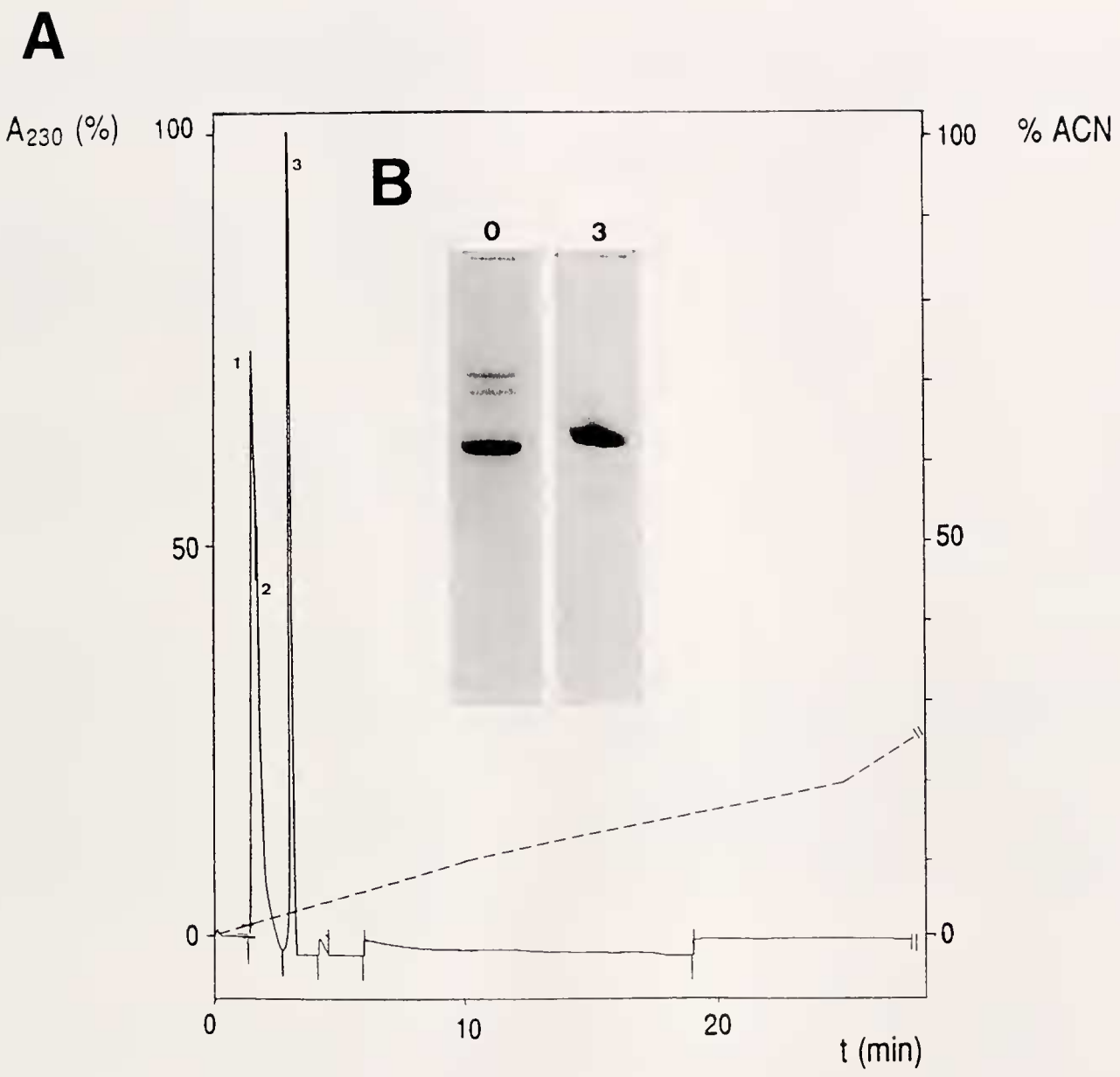


Figure 1. (A) Acetic acid (5%)—urea (2.5 *M*)—PAGE analysis of (1) chicken erythrocyte histones, (2) nuclear sperm proteins from the mussel *Mytilus californianus*, (3) nuclear sperm proteins from the ascidian tunicate *Styela plicata*, (4) nuclear sperm proteins from the lancelet *Branchiostoma floridae*, and (5) salmine (protamine). Direction of electrophoresis is from top (+) to bottom (−). (B) Acetic acid (5%)—urea (6 *M*) (first dimension)—SDS (second dimension) two-dimensional gel electrophoresis of the proteins shown in (A) lane 4.

like proteins (PL) to protamines (P), depending on the organism (Bloch, 1969, 1976). Those organisms that retain the somatic-histone type of proteins often contain sperm-specific histones (H1 and/or H2B) and will be referred to as (H1 type) in this paper.

Although the nature of the compositional protein heterogeneity of the sperm is unclear, from an evolutionary point of view protamines are more specialized proteins and therefore might be expected to have appeared in more evolved organisms, whereas the less specialized histones would be present in more primitive species. From this perspective, the data available on the taxonomic distribution of protamines within and among different taxonomical groups still remains a puzzle. A clear example of this situation has been recently exemplified in the bony fish (Saperas *et al.*, 1993a, b). The organisms within this group have sperm cells with nuclear protein compositions including all the protein types (H, H1, PL, P) described earlier.

The problem of nuclear protein heterogeneity within bony fish has been linked to the controversial evolutionary origin of protamines in fish (see Oliva and Dixon, 1991,



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G/P R S R S R S A S

Figure 2. (A) Reverse phase HPLC fractionation of the whole nuclear protein extract (0.4 *N* HCl) from the sperm of *Branchiostoma floridae* on a 5 μm, Spherisorb C_{18}, ODS2 (4.6 × 150 mm) column. A_{230} = absorbance at 230 nm. ACN = acetonitrile. No proteins could be detected in peaks 1 and 2. (B) Acetic acid (5%)—urea (6 *M*) PAGE of 0: starting nuclear protein extract; 3: protein fraction eluted in peak 3 of Figure 2(A). (C) Amino acid sequence of the N-terminal region of the protein shown in (B) lane 3.

Table 1

Amino acid analysis (mol %) of the nuclear sperm-specific protein of Branchiostoma floridae *PL(BF) in comparison to the PL-III proteins of* Mytilus trossulus *PL-III (MT) (Mogensen et al., 1991) and* Macoma nasuta *PL-III (MN) (Austió, 1988)*

	PL(BF)	PL III (MT)	PL III (MN)
Lys	24.7	24.0	25.2
His	—	—	0.2
Arg	25.3	27.5	27.9
Asx	—	—	3.5
Thr	—	3.7	—
Ser	16.5	17.7	28.7
Glx	—	—	0.9
Pro	5.6	5.1	0.6
Gly	6.1	6.9	1.8
Ala	21.7	14.1	9.5
Cys	—	—	—
Val	—	0.9	0.4
Met	—	—	0.1
Ile	—	—	0.5
Leu	—	—	0.5
Tyr	—	—	0.1
Phe	—	—	0.2

for a discussion). Thus it seemed timely to analyze the nuclear sperm-specific proteins of more primitive organisms related to early vertebrate evolution. We have characterized the sperm-specific proteins of an agnathan (*Petromyzon marinus*) and a cephalochordate (*Branchiostoma floridae*) and compared them to the nuclear sperm-specific proteins of different groups of deuterostomes. The possible evolutionary significance of these data is discussed.

Materials and Methods

Living organisms

Adult males of *Branchiostoma floridae* (Hubbs, 1922) were collected as described elsewhere (Holland and Holland, 1989).

Male lampreys, *Petromyzon marinus* (Linnaeus, 1758), were collected during their upstream (prespawning) migration in the watersheds of Lake Ontario, Canada in November 1990, 1991, 1992. The animals were held in the laboratory at Scarborough Campus until sexually mature and then the milt was released by abdominal massage into test tubes for immediate freezing at −70°C or for fixation for electron microscopy. *Lampretra richardsoni* were collected in Southwestern British Columbia.

Nuclei preparation

Nuclei were isolated from ripe sperm cells as described elsewhere (Saperas *et al.*, 1993a).

Protein extraction

Crude protein extracts were obtained by homogenization of the nuclei in 0.4 *N* HCl followed by precipitation of the soluble fraction in 6 volumes of acetone overnight at 4°C.

Reduction of disulfide linkages

S-S linkages were reduced under denaturing conditions as described by Kuehl (1979). In brief, the proteins, at a concentration of 1 mg/ml in 6 *M* urea, 20 m*M* Tris-HCl pH 7.6, were reduced in the presence of 8% β-mercaptoethanol for 3 h at room temperature.

Protein fractionation and purification

Ionic exchange chromatography using carboxymethyl cellulose (Whatman CM52) and reverse phase HPLC [on either C_4 or C_{18} (Vydac)] were carried out as described elsewhere (Saperas *et al.*, 1992).

Amino acid analysis and protein sequence determination

Amino acid analyses were carried out on an Applied Biosystems (ABI) model 420 A derivatizer-analyzer system. The hydrolysis of the protein was carried out in a gas-phase 6 *N* HCl and 1% phenol under an argon atmosphere at 165°C. The N-terminal sequence of the proteins was determined by automated Edman degradation on an Applied Biosystems (ABI) model 470A protein sequencer using the standard program 03 rpth. The phenylthiohydantoins were analyzed on an Applied Biosystems (ABI) model 120A on line HPLC system using a C_{18} Brownlee column (2.1 × 220 mm).

Gel electrophoresis

Acetic acid (2.5 or 6 *M*) urea polyacrylamide gel electrophoresis (PAGE) was performed as described elsewhere (Saperas *et al.*, 1992). Two-dimension gel electrophoresis using acetic acid-urea or acetic acid-urea-triton X-100 for the first dimension and sodium dodecyl sulfate for the second, was carried out as described elsewhere (Saperas *et al.*, 1992).

Electron microscopy

Transmission electron microscopy of the *Petromyzon* sperm was carried out at the Service of Electron Microscopy of the University of Barcelona. The samples were prepared as described elsewhere (Saperas *et al.*, 1993a). Electron microscopy analysis of *Branchiostoma* was carried out by Dr. N. Holland of the Marine Biology Research Division at Scripps Institution of Oceanography (La Jolla, California) as described in Holland and Holland (1989).

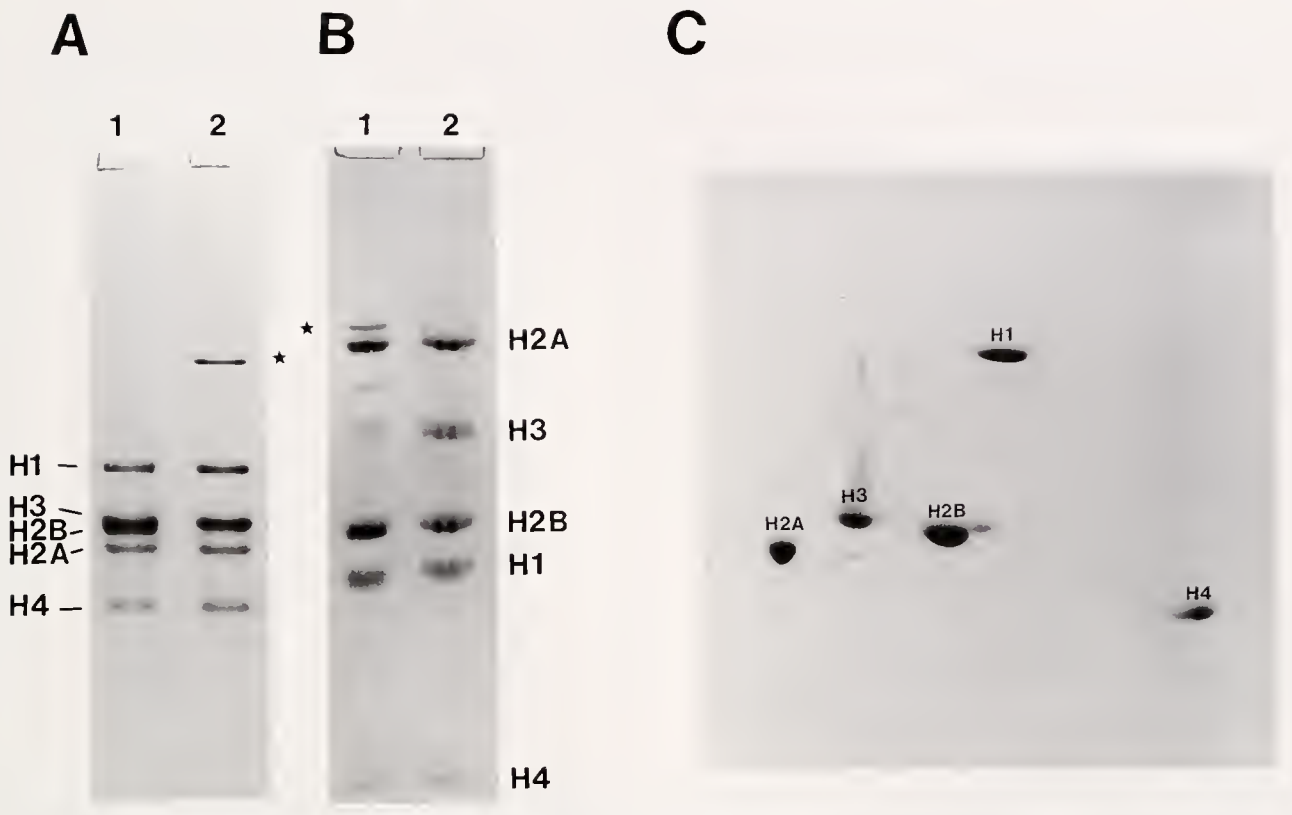

Figure 3. (A) Analysis of a whole nuclear protein extract from the sperm of *Petromyzon marinus* in acetic acid (5%)—urea (2.5 *M*) (1) after and (2) before reduction with β-mercaptoethanol. (B) Acetic acid (5%)—urea (6 *M*)—triton X-100 (6 m*M*) PAGE of a whole nuclear protein extract from the sperm of *Petromyzon marinus* (1) before and (2) after treatment with β-mercaptoethanol. (C) Two-dimensional PAGE in acetic acid-urea-triton X-100 as in (B) (first dimension), SDS (second dimension). The asterisks indicate the position of the histone H3 dimers.

Results

The nuclear sperm-specific proteins of the cephalochordate Branchiostoma floridae

The electrophoretic analysis of a whole protein extract from the nuclei of the sperm of *B. floridae* is shown in Figure 1. The analysis is shown in comparison to somatic histones from chicken erythrocyte (lane 1) and to proteins of the PL-type from a mussel (*Mytilus edulis*, lane 2) and from an ascidian tunicate (*Styela plicata*, lane 3). Several minor bands with electrophoretic mobility similar to that of histones (both in urea-acetic acid, Fig. 1A, or in SDS gels, Fig. 1B) coexist with a major band of higher electrophoretic mobility. The protein corresponding to this major band was purified by reverse phase HPLC (see Fig. 2). The compositional amino acid analysis of this protein is given in Table I. Such composition is clearly indicative of the highly specialized nature of this protein. Only six amino acids are present, four of which (Lys, Arg, Ser, and Ala), account for 88% of the overall amino acid composition. The basic amino acids alone represent 50% of this composition. The number of constitutive amino acids estimated from the electrophoretic mobility of this protein in polyacrylamide urea acetic acid gels (Colom and Subirana, 1979; Ausio and Subirana, 1982a; Daban *et al.*, 1991) was 85 ± 5. Despite the small amount of purified protein available, it was still possible to establish the amino acid sequence of the first nine amino acids of the N-terminal region of this protein. The results are shown in Figure 2C. G/P at the N-terminus of this sequence indicates an almost identical recovery of these two amino acids in the first cycle of Edman degradation. This is most likely due to the existence of some protein microheterogeneity.

The nuclear sperm-specific proteins of the Agnathan Petromyzon marinus

A whole nuclear protein (0.4 *N* HCl extract) from the sperm of *Petromyzon marinus* is shown in Figure 3. Based on their relative electrophoretic mobility in polyacrylamide gels containing either urea-acetic acid, urea-acetic acid-triton X-100 or SDS (Fig. 3A, B, C), the proteins

Table II

Amino acid composition (mol %) of the sperm histones of the lamprey Petromyzon marinus *(PM) in comparison to the somatic histones from calf thymus (CT) (Mayes and Johns, 1982)*

	H1		H2A		H2B		H3		H4	
	PM	CT	PM	CT	PM	CT	PM	CT	PM	CT
Lys	27.6	26.8	9.7	10.2	14.1	14.1	8.3	10.0	10.4	11.4
His	—	—	1.6	3.1	3.3	2.3	1.7	1.7	1.8	2.2
Arg	2.2	1.8	9.8	9.4	8.2	6.9	13.8	13.0	13.4	12.8
Asx	3.9	2.5	6.3	6.2	4.8	5.0	4.2	4.2	4.8	5.2
Thr	3.6	5.6	3.2	3.9	6.6	6.4	7.5	6.8	6.7	6.3
Ser	7.3	5.6	5.2	3.4	10.3	10.4	4.5	3.6	2.0	2.2
Glx	4.7	3.7	9.4	9.8	6.4	8.7	12.0	11.6	6.5	6.9
Pro	9.7	9.2	4.1	4.1	6.2	4.9	4.5	4.6	1.4	1.5
Gly	4.0	7.2	11.4	10.8	5.9	5.4	5.6	5.4	16.8	14.9
Ala	21.8	24.3	13.1	12.9	11.6	10.8	13.3	13.3	7.9	7.7
Cys	—	—	—	—	—	—	0.6	1.0	—	—
Val	6.8	5.4	7.2	6.3	6.0	7.5	4.2	4.4	8.1	8.2
Met	—	—	0.5	—	2.1	1.5	0.8	1.1	0.2	1.0
Ile	1.1	1.5	3.7	3.9	5.8	5.1	4.7	5.3	5.5	5.7
Leu	5.3	4.5	11.9	12.4	4.5	4.9	8.9	9.1	8.6	8.2
Tyr	0.8	0.9	2.3	2.2	2.8	4.0	2.2	2.2	3.6	3.8
Phe	1.1	0.9	0.9	0.9	1.6	1.6	3.3	3.1	2.1	2.1

solubilized by 0.4 *N* HCl behave as the typical somatic nucleosomal histones of most eukaryotes. Under the acidic conditions of this method of extraction, about 70% of histone H3 is present in a dimer conformation (see Fig. 3A, lane 2, 3B, lane 1). This dimer form can be easily converted to the monomer form by treatment with β-mercaptoethanol (Fig. 3A, lane 1, 3B, lane 2). The absence of aggregates larger than dimers, together with the amino acid composition of this histone fraction (Table II) indicate that H3 from *P. marinus* contains only one cysteine residue per molecule as it occurs with most somatic histone H3 proteins (Klyszejko-Stefanowicz *et al.*, 1989). To date, somatic type histones (type H) have been found in the sperm of organisms as diverse as the horseshoe crab (*Limulus polyphemus*) (Muñoz-Guerra *et al.*, 1982a), the frog (*Rana catesbiana*) (Kasinsky *et al.*, 1985), and in some fish such as the goldfish (*Carassius auratus*) (Muñoz-Guerra *et al.*, 1982b), just to mention a few examples (see also Kasinsky, 1989, for a more extensive review).

Figure 4 shows a reverse phase HPLC fractionation of the 0.4 *N* HCl nuclear extract. The individual fractions purified in this way were subjected to amino acid analysis and the compositions are shown in Table II. These results corroborate the true histone nature of the basic proteins from the nucleus of the sperm of *Petromyzon marinus*.

Light and electron microscopy studies

Figure 5A, B, C shows a light microscopy comparison of the sperm of a lancelet (*Branchiostoma floridae*) and two lampreys (*Petromyzon marinus* and *Lampetra richardsoni*). The head of the sperm in the lancelet and in the lamprey *Petromyzon* is rounded, whereas in contrast the sperm of *Lampetra* has an elongated shape as previously reported (Stanley, 1967). The relevance of this observation will be discussed later. The micrographs can also be used to assess the extent of purity of the sperm samples used in the protein preparations.

Figure 6(1) shows an electron micrograph of a partial view of the sperm head of *Petromyzon marinus*. Although for the purposes of this work we are primarily interested in the fine structure of the chromatin complexes within the nucleus, several distinctive regions of the sperm head can still be distinguished clearly in this micrograph. These characteristic structures—acrosomal vesicles (AC), endonuclear canal (EC), and the central fiber (CF)—have also been described in other lampreys (Stanley, 1967; Jamieson, 1984), and they are very similar in all of them except for the elongated shape of the nucleus of *Lampetra* (Stanley, 1967).

At the chromatin level, the fine structure of the nucleus reveals a closely packed granular organization [Fig. 6(2)] which most likely corresponds to the same granular organization observed by Stanley (1967) in *Lampetra*. In the latter case the superficial granular appearance is due to a filamentous fiber structure that is most likely also present in *Petromyzon*. In fact the diameter of the granules observed [Fig. 6(2)] is 3.0 ± 0.5 nm. This diameter is very close to the 2.37 nm observed by low angle X-ray diffraction for the nucleoprotamine fibers of the sperm of

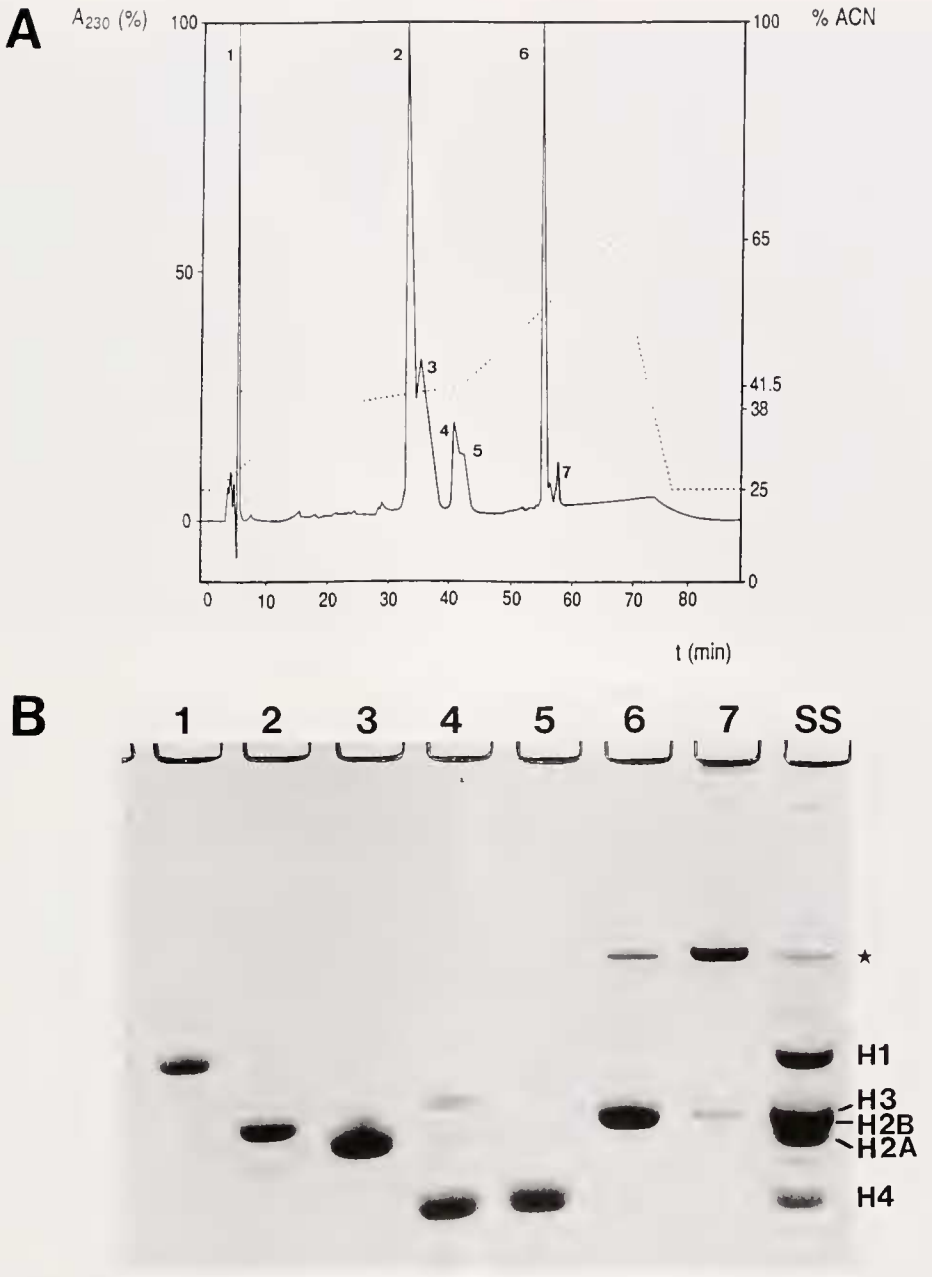

Figure 4. (A) Reverse phase HPLC fractionation of a whole nuclear protein extract from the sperm of *Petromyzon marinus* on a 5-μm, Vydac C_{18} (4.6 × 250 mm) column. (B) Electrophoretic analysis on acetic acid (5%)—urea (2.5 *M*) PAGE of the corresponding elution peaks of Figure 4(A). SS: starting sample. The asterisk indicates the position of the H3 dimers.

Mytilus (Ausió and Subirana, 1982b). This kind of chromatin organization is quite different from that observed in the lancelet [Fig. 6(4)] (Holland and Holland, 1989). In this case as spermiogenesis proceeds, particles 30 nm in diameter (fibers?) coalesce into granules of about 60 nm in diameter in the mature spermatozoa [Fig. 6(3) and 6(4), Holland and Holland, 1989]. These granules have an enormous resemblance to the 40–70 nm granules observed by electron microscopy in the sperm of *Mytilus* (Longo and Dornfeld, 1967).

Discussion

Compositional heterogeneity of the sperm-specific nuclear proteins in deuterostomes

To ascertain the possible significance and evolutionary implications of the protein composition from the nucleus

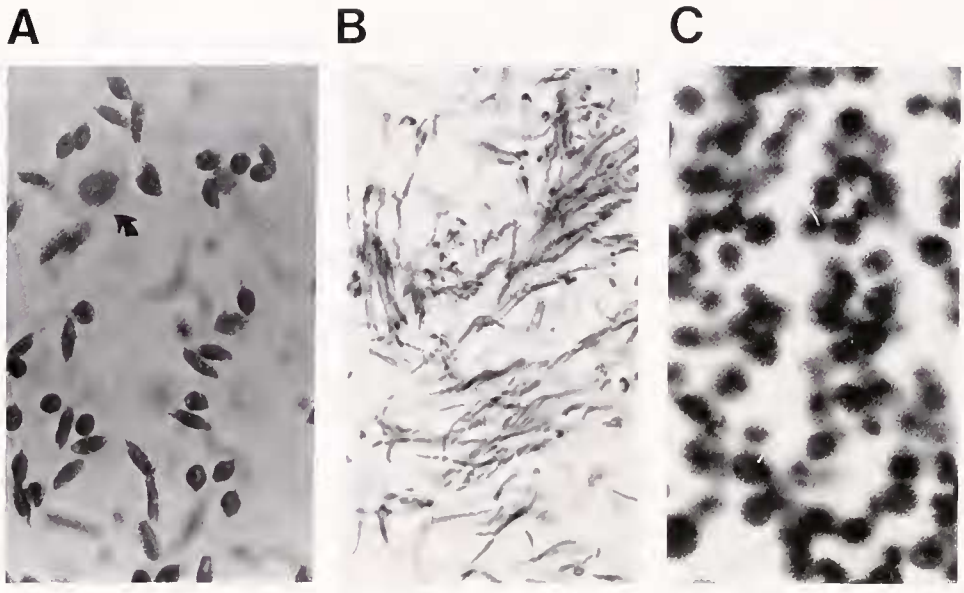

Figure 5. (A) Light microscope phase contrast micrograph of the sperm suspension of *Petromyzon marinus* used to prepare the sperm-specific nuclear proteins shown in Figure 3 and Figure 4. About 2–3% of the sample consisted of erythrocyte contamination (arrow). (B) Light microscope Feulgen stain of a section of an embedded sperm sample from *Lampetra richardsoni testis*. (C) Light microscope Feulgen stain of a sperm suspension from *Branchiotoma floridae*. The magnification was ×1000 in every case.

of the sperm of *Branchiostoma floridae* (Cephalochordata) and *Petromyzon marinus* (Agnatha), the analyses must be carried out in comparison to the different groups of deuterostomes for which the sperm-specific protein composition is already well established. At present some information is available about the chromosomal proteins of the sperm of echinoderms, ascidian tunicates, and fish (see also Fig. 7 and Table III).

The sperm-specific proteins of the echinoderms have been extensively characterized. They consist basically of histone variants (type H of the classification outlined in the Introduction), although in most cases highly specialized sperm-specific histone H1 or H2B fractions have been described (type H1) (see Fig. 7, lanes 1, 2, 3). Thus, within the Subphylum Asterozoa, several representative organisms of the Class Stelleroidea have been analyzed (see also Fig. 7, lane 1) (Subirana and Palau, 1968; Strickland *et al.*, 1980; Zalenskaya *et al.*, 1980). In the sperm of these organisms the histone H1 fraction is different from its somatic counterpart, whereas the core histones (H2A, H2B, H3, H4) exhibit an electrophoretic mobility in PAGE that is indistinguishable from that of the somatic core histones (Zalenskaya *et al.*, 1980). Within the Subphylum Echinozoa, several organisms within each of the classes Echinoidea and Holothuroidea have been characterized (see Fig. 7, lane 2). In the Echinoidea, most of its representative organisms have highly specialized histone H1 and H2B variants (Strickland, W. N. *et al.*, 1980a, b, 1982a, b; Brandt *et al.*, 1979; Giancotti *et al.*, 1980; Zalenskaya and Zalensky, 1980; Imschenetzky *et al.*, 1984). These variants exhibit modified N- or C-terminal regions, which are often characterized by the presence of very characteristic repetitive (tetra or penta)-peptides (Strickland, W. N. *et al.*, 1977; Strickland, M. *et al.*, 1977, 1978; Strickland, W. N. *et al.*, 1980a, b) (see also von Holt *et al.*, 1984, and Poccia, 1991, for more detailed reviews). Within the organisms of the Class Holothuroidea, *Holothuria tubulosa* contains a highly specific heterogeneous histone H1 in addition to the somatic counterpart (Phelan *et al.*, 1972). In addition to the histone complement, all the organisms characterized so far within this class also contain an additional protein with higher electrophoretic mobility (~80 amino acids) (see Fig. 7, lane 3) (Subirana, 1970; Zalenskaya *et al.*, 1980). The sequence of one of these proteins was determined recently (Prats *et al.*, 1989).

In most of the ascidian tunicates, the basic proteins from the nucleus of the sperm consist of a major protamine-like component (PL) that replaces the nucleosomal histones (Chiva *et al.*, 1990, 1992; Saperas *et al.*, 1992) (Fig. 7, lane 4). This protein consists of approximately 145 amino acids and has an amino acid composition and a trypsin-resistant peptide (Saperas *et al.*, 1992) that resembles those of PL-I proteins of mollusks (Ausió *et al.*, 1987; Jutglar *et al.*, 1991). In at least one genus of ascidian tunicates (genus = *Styela*) an additional PL with fewer amino acids (80–85) is also present (see Fig. 7, lane 5, and Fig. 1, lane 3). The amino acid composition of this latter protein resembles that of

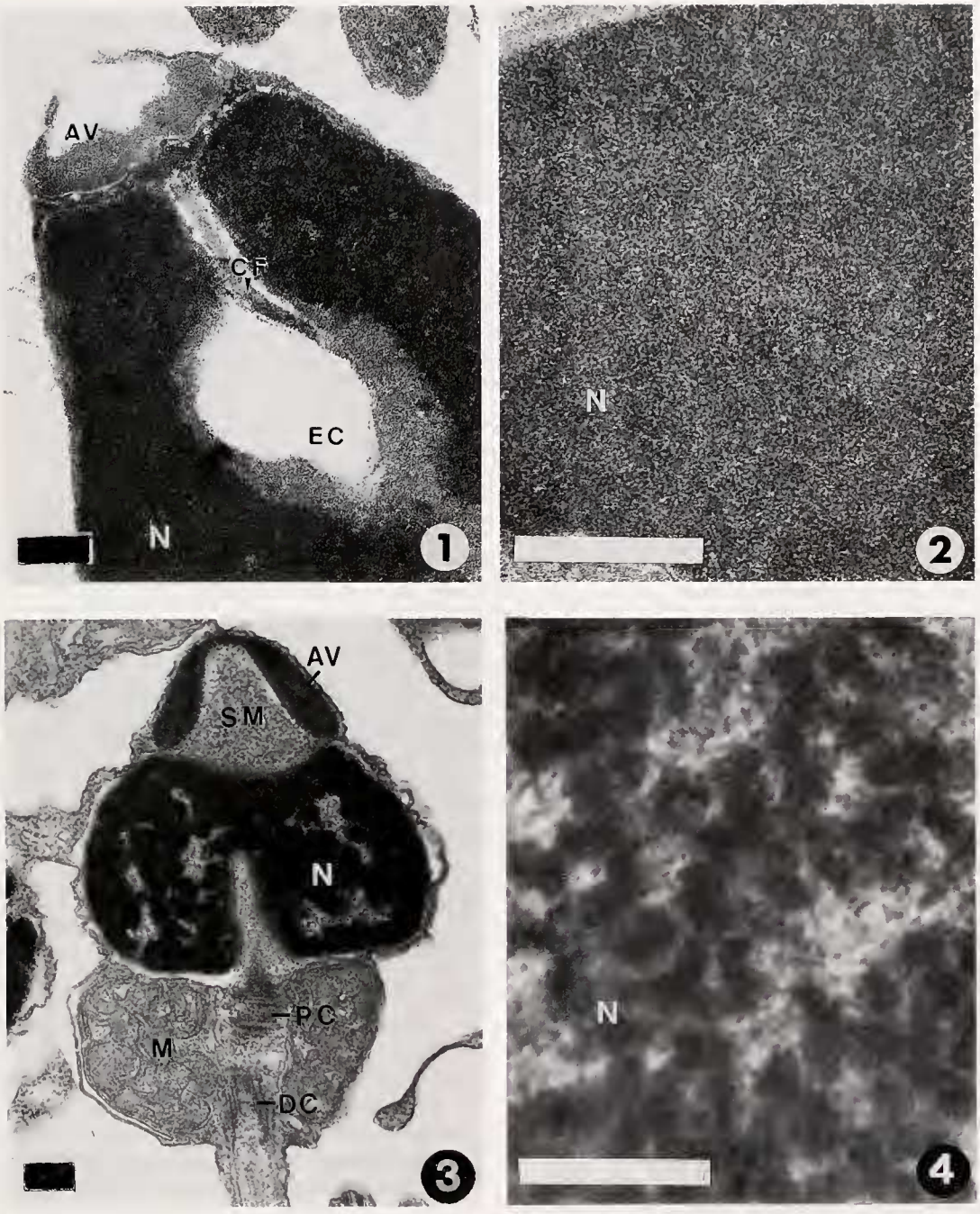

Figure 6. (1) Electron micrograph of the apical region of the sperm of *Petromyzon marinus*, showing the acrosomal vesicle (AV), the endonuclear canal (EC) and the central fiber (CF) and the nucleus (N) (×53,000); (2) Detail of the nucleus of the sperm of *P. marinus* to show the fine structure of chromatin (×140,000). (3) Electron micrograph of a mature sperm cell from *Branchiostoma floridae* acrosomal vesicle (AV); distal centriole (DC); mitochondria (M); nucleus (N); proximal centriole (PC); and subacrosomal material (SM) (×37,000). (4) Detail of the nucleus of the sperm of *Branchiostoma floridae* (×140,000). All scale bars are 200 nm.

protamines from teleost fish (Saperas *et al.*, 1992). However, it is not clear whether there is an evolutionary relation between these proteins or whether the presence of the additional PL protein only represents a particular solution to the process of chromatin compaction during spermatogenesis for the members of this genus.

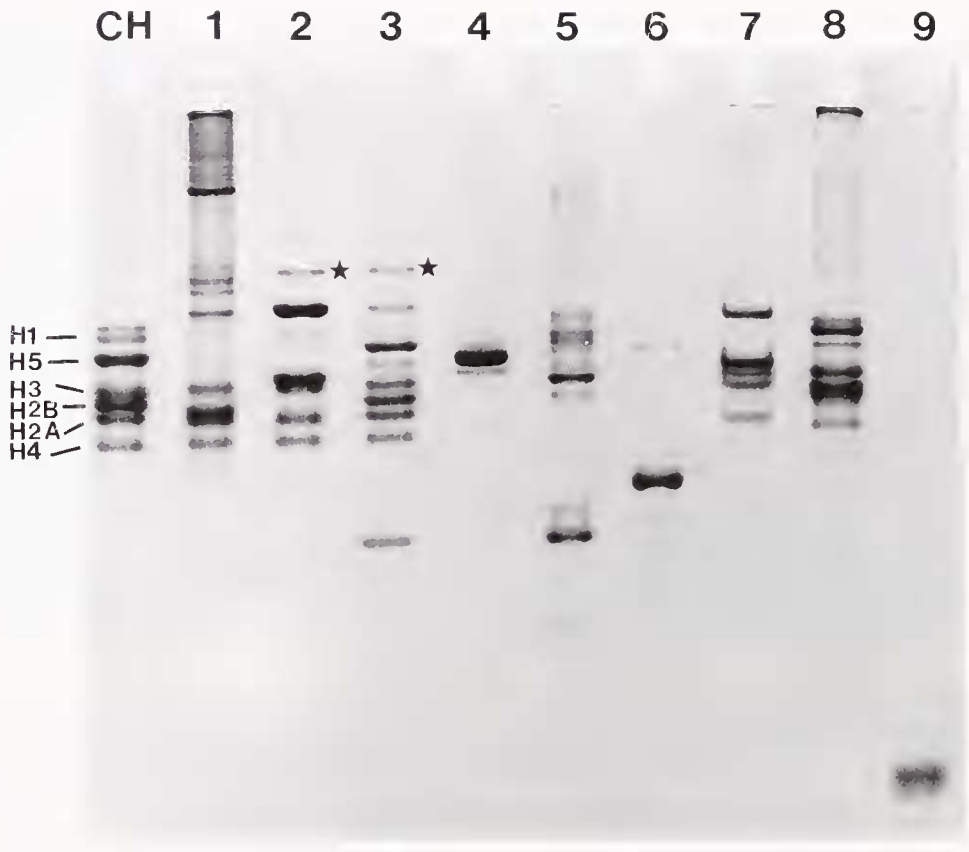

Figure 7. Acetic acid (5%)—urea (2.5 *M*) PAGE of whole nuclear protein extracts from the sperm of several groups of deuterostomes: CH: Chicken erythrocyte histones used as standard; (1) starfish *Pisaster ochraceus;* (2) sea urchin *Strongylocentrotus purpuratus;* (3) sea cucumber *Thyone briareus;* (4) ascidian tunicate *Phallusia mammillata.* The proteins shown in this lane are a 0.4 *N* HCl protein extract from nuclei that had been previously treated with 35% acetic acid in order to remove the residual histones. (5) ascidian tunicate *Styela plicata;* (6) lancelet *Branchiostoma floridae;* (7) lamprey *Petromyzon marinus;* (8) fish *Trigla lucerna;* (9) commercial salmon fish protamine (salmine) *Onchorhyncus* sp. The asterisks (*) point to the histone H3-H3 dimers resulting from oxidation of the cysteine residues.

The only cephalochordate that has been characterized thus far from the point of view of its nuclear sperm proteins is that described in the present work. As has already been mentioned in the preceding section, the major nuclear protein of the sperm of *Branchiostoma floridae* is a protamine-like (PL) protein (Fig. 2, Fig. 7, lane 6). The amino acid composition of this protein (Table I) is different from that of fish protamines and from that of the smaller PL present in *Stylelidae.* [P2c (SP) in Table III.] It is very similar, however, to PL-III proteins (Ausió, 1986) from mollusks (Table I). In addition, the amino acid sequence of the first nine N-terminal amino acids (Fig. 2C) with its repetitive "RS" motif largely resembles the primary structure of the N-terminal regions of PL in mollusks (Daban, 1991; Carlos *et al.,* 1993), which also exhibit this kind of repetitive structure. An "RS" repetitive motif is also present in the N-terminal region of the bird protamines (Oliva and Dixon, 1989) and to a different extent is also present in other vertebrate protamines (Kasinsky, 1989; Oliva and Dixon, 1991).

From an evolutionary perspective (Fig. 8), the finding that somatic-like histones are present in the sperm of the agnathan *Petromyzon marinus* is quite unexpected. It is surprising, considering that substitution of histones by more specialized proteins (PL) has already been known to occur in tunicates and in cephalochordates as discussed above. Nevertheless, a similar situation is also found in teleost fish (see Fig. 8), where an apparently random distribution of histones, protamine-like, and protamine proteins is observed among different orders, families, and genera (Saperas *et al.,* 1993a, b).

We envisage two alternative possibilities to explain these observations. The first is that during the evolution of the sperm-specific proteins within the Subphylum Vertebrata (see Fig. 8A), histones represent the early proteins present in the nucleus of the sperm that, during the evolution of

Table III

Amino acid composition (mol %) of different nuclear sperm proteins: histone III from starfish Aphelasterias japonica *III (AJ) (Zalenskaya* et al., *1980), histone III from sea urchin* Parechinus angulosus *III (PA) (Brandt et al., 1979), PL(fo) protein from sea cucumber* Holothuria tubulosa *PL(fo) (HT) (Subirana, 1983), proteins P1, P2 from ascidian tunicate* Styela plicata *P1, $P2_c$ (SP) (Saperas* et al., *1992), protein PL from lancelet* Branchiostoma floridae *PL(BF) (this work), and the protamine salmine SL (SI) from* Salmo irideus *(Ando and Watanabe, 1969)*

	H1 (AJ)	H1 (PA)	$PL^{(fo)}$ (HT)	P1 (SP)	$P2_c$ (SP)	PL (BF)	SL (SI)
Lys	24.5	25.1	17.0	14.3	17.4	24.7	—
His	0.8	0.9	—	1.1	—	—	—
Arg	14.5	9.1	25.9	32.5	50.4	25.3	65.6
Asx	1.6	1.8	—	4.6	1.2	—	—
Thr	2.4	2.0	2.1	1.2	3.1	—	—
Ser	9.5	7.2	9.4	4.6	1.1	16.5	12.5
Glx	3.0	2.3	2.9	2.2	—	—	—
Pro	4.6	8.5	6.5	1.4	—	5.6	9.4
Gly	4.6	4.4	—	13.2	18.7	6.1	6.2
Ala	31.4	29.4	29.5	7.1	3.3	21.7	—
Cys	—	—	—	0.7	—	—	—
Val	1.7	4.0	5.0	4.6	—	—	6.2
Met	tr.	1.7	—	1.4	0.7	—	—
Ile	0.8	0.8	1.3	2.4	—	—	—
Leu	0.7	2.1	—	4.5	3.3	—	—
Tyr	tr.	0.8	—	2.2	0.7	—	—
Phe	tr.	0.4	—	2.2	—	—	—
Trp	—	—	—	—	—	—	—

tr: trace amounts.

this group, have been progressively replaced by more specialized proteins (protamine-like and protamines). Other subphyla such as Cephalochordata (lancelet) and Urochordata (tunicates) might have had different (although related) evolutionary pathways, and the histones in these later two groups might have been lost during the diversification of the Phyla Chordata and Echinodermata.

A second evolutionary alternative for the evolution of the chromosomal sperm proteins of deuterostomes is shown in Figure 8B. Accordingly, histones would have been replaced early by a protamine-like (PL) ancestor in chordates. This protein would have given origin to the PL proteins found in urochordates and in cephalochordates and maybe, to the fish protamines. In the lamprey the gene (or its expression) for such highly specialized chromosomal sperm proteins might have been lost. Obviously these are idealized models that represent two extreme alternatives that might be useful for the understanding of the evolutionary complexity of these proteins in deuterostomes. These hypotheses are difficult to test, especially if one considers the quickly changing ideas about evolution of deuterostomes (Stock and Whitt, 1992; Forey and Janvier, 1993; Conway Morris, 1993).

Despite all this, a quick inspection of Figure 8 reveals that the evolutionary trend in deuterostomes (thick arrow in Fig. 8) is to acquire highly specialized protamines (P) that replace the somatic-like histones. As discussed below, this might be closely related to the evolution of fertilization mechanisms in the deuterostomes (Kasinsky, 1989).

Shape of the sperm-nucleus and chromatin organization in Branchiostoma *and* Petromyzon: *further evolutionary considerations*

Figure 5 shows the morphology of the spermatozoa of two lampreys, *Petromyzon marinus* and *Lampetra planeri*. As mentioned previously, the morphology of the sperm of these closely related species is quite different despite the fact that both seem to have a very similar chromatin composition and organization. Furthermore, both organisms are anadromous and have external fertilization, although, as Jamieson (1991, p. 63) points out, in *P. marinus* "the cloacal tube (penis) of the male is fitted closely to the female pore and eggs are fertilized as they leave the body in what appears to be neither truly external nor internal fertilization (Breder and Rosen, 1966)." These observations seem to contradict (at least in this particular instance), the hypothesis that tries to correlate sperm shape with fertilization (internal *versus* external) (Baccetti and Afzelius, 1976; Jamieson, 1991). It also contradicts the hypothesis put forward by Nandi *et al.* (1979) that suggested that the protein composition of chromatin in the sperm (histones *versus* protamines) might depend on the physiochemical conditions (such as salinity) of the medium in which fertilization occurs. Considering that histones are the major protein constituents of the sperm chromatin in *Petromyzon marinus*, the structural organization of the 3-nm chromatin fibers in the mature sperm (as visualized in the electron microscope, Fig. 6, lane 2)

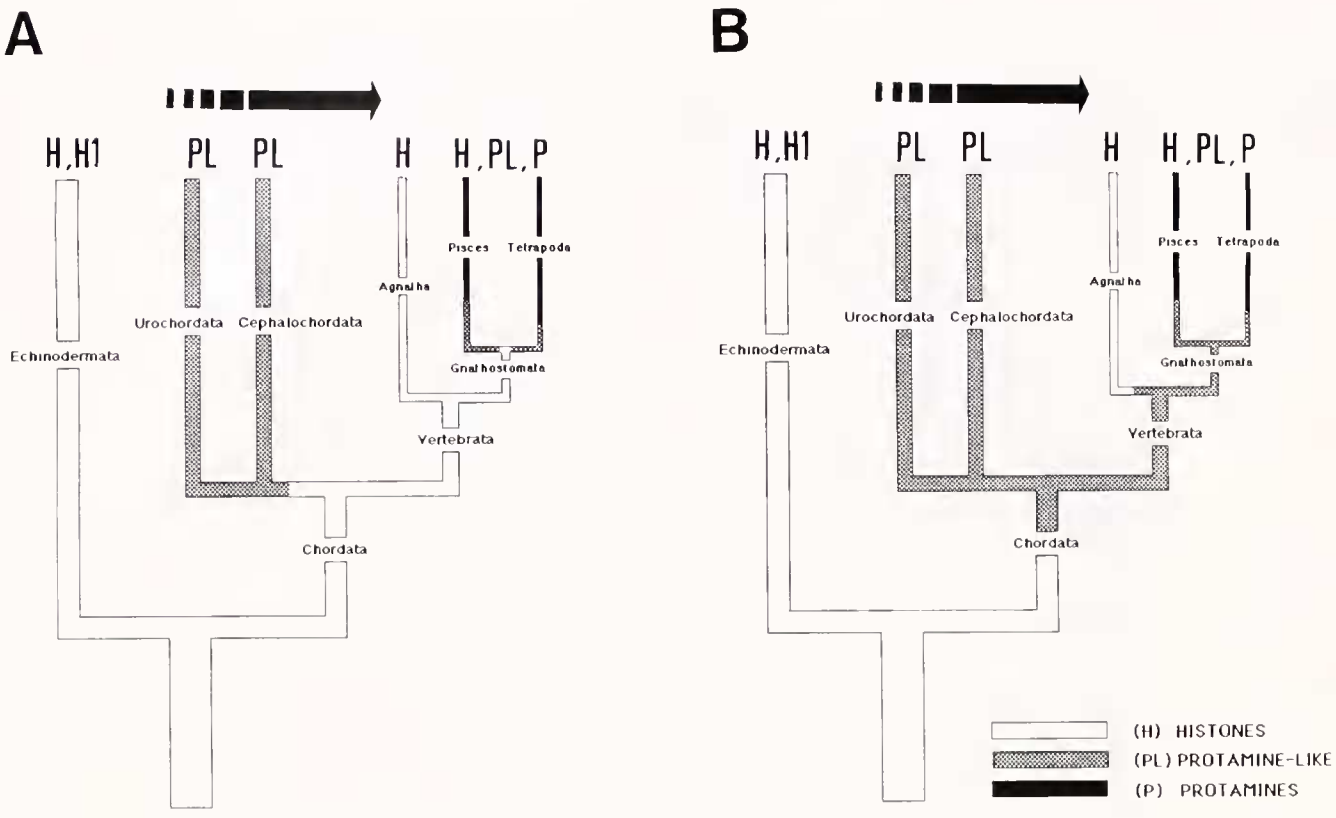

Figure 8. Schematic representation of the phylogenetic relationships of the main groups of deuterostomes, adapted from Stock and Whitt (1992) and from Brusca and Brusca (1990). H: mature sperm containing only histones; H1: mature sperm containing histones with a highly specialized histone H1; PL: mature sperm containing mainly intermediate protamine-like (PL) proteins. P: mature sperm containing protamines. The arrow indicates the progressive transition from the histone type (H) to the protamine type (P) as a result of evolution.

appears to be unique. Association of core histones (H2A, H2B, H3, and H4) with the DNA usually leads to chromatin fibers consisting of randomly spaced nucleosome particles of about 10 nm in diameter. In the presence of histones of the H1 family (that bind to the internucleosomal linker DNA), the fiber compacts into a higher order structure giving rise to fibers of about 30 nm in diameter (see Van Holde, 1988, for a review). Fibers of a similar diameter have also been observed in the chromatin of the sperm nucleus of different organisms containing histones or other sperm-specific proteins (Casas *et al.*, 1993).

The chromatin arrangement observed in *Petromyzon* is very similar to that recently described in the sperm of the fish *Mullus surmuletus* (Saperas *et al.*, 1993b). In the latter case, however, the protein composition of chromatin consists of only two PL proteins and no histones. Therefore, the finding of a 3-nm fiber in *Petromyzon* raises the very challenging possibility that histones could organize chromatin in alternative structures to nucleosomes. In the case of *Branchiostoma floridae*, the sperm shape (Fig. 5C) (Holland and Holland, 1989) is in good agreement with that of a primitive sperm (Baccetti and Afzelius, 1976). The organization of chromatin, as visualized by electron microscopy [Fig. 6(4)] (Holland and Holland, 1989), is similar to that observed in other invertebrate organisms with a similar protein composition (Longo and Dornfeld, 1967; Casas *et al.*, 1993).

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